PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 :
A61K 37/00, 39/00, C07H 15/12
C07H 17/00, C07K 3/00, 13/00
C07K 15/00, 17/00

A1

(11) International Publication Number: WO 93/16712

(43) International Publication Date: 2 September 1993 (02.09.93)

(21) International Application Number: PCT/US93/01734

(22) International Filing Date: 25 February 1993 (25.02.93)

(30) Priority data:
07/842,077 26 February 1992 (26.02.92) US

(60) Parent Application or Grant
(63) Related by Continuation
US 07/842,077 (CIP)
Filed on 26 February 1992 (26.02.92)

(71) Applicant *(for all designated States except US)*: THE SCRIPPS RESEARCH INSTITUTE [US/US]; 10666 North Torrey Pines Road, La Jolla, CA 92037 (US).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)* : RUGGERI, Zaverio, M. [IT/US]; 6633 Avenida de las Pescas, La Jolla, CA 92037 (US). WARE, Jerry, L. [US/US]; 2119 Summerhill Drive, Encinitas, CA 92024 (US).

(74) Agents: BARRON, Alexis et al.; Synnestvedt & Lechner, 2600 One Reading Center, 1101 Market Street, Philadelphia, PA 19107-2950 (US).

(81) Designated States: AT, AU, BB, BG, BR, CA, CH, CZ, DE, DK, ES, FI, GB, HU, JP, KP, KR, LK, LU, MG, MN, MW, NL, NO, NZ, PL, PT, RO, RU, SD, SE, SK, UA, US, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, SN, TD, TG).

Published
With international search report.

(54) Title: MUTANT GPIbα FRAGMENTS AND RECOMBINANT EXPRESSION THEREOF

(57) Abstract

A polypeptide patterned on a fragment of wild type glycoprotein Ibα having a predetermined affinity for von Willebrand factor, said polypeptide having a modified sequence of amino acids relative to that of said fragment and an increased binding affinity, relative to said predetermined affinity, for von Willebrand factor, for example, wherein said modification comprises replacement of one or more amino acid residues of said fragment with one or more amino acid residues found at the equivalent sequence positions of glycoprotein Ibα as isolated from one or more humans with platelet-type von Willebrand disease; and, in addition, a process for producing from DNA encoding glycoprotein Ibα, or a fragment thereof, an aforementioned polypeptide which process includes, for example, use of a DNA sequence in which one or more wild type codons thereof are replaced by codons specifying one or more amino acid mutations found in the glycoprotein Ibα DNA sequence of one or more patients having platelet-type von Willebrand disease; and, in addition, a therapeutic composition which is effective in treating or inhibiting thrombosis comprising a pharmaceutically acceptable carrier and a polypeptide of the invention, and also a method of treating or inhibiting thrombosis in a patient which comprises administering to such patient an effective amount of said therapeutic composition.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	FR	France	MR	Mauritania
AU	Australia	GA	Gabon	MW	Malawi
BB	Barbados	GB	United Kingdom	NL	Netherlands
BE	Belgium	GN	Guinea	NO	Norway
BF	Burkina Faso	GR	Greece	NZ	New Zealand
BG	Bulgaria	HU	Hungary	PL	Poland
BJ	Benin	IE	Ireland	PT	Portugal
BR	Brazil	IT	Italy	RO	Romania
CA	Canada	JP	Japan	RU	Russian Federation
CF	Central African Republic	KP	Democratic People's Republic of Korea	SD	Sudan
CG	Congo			SE	Sweden
CH	Switzerland	KR	Republic of Korea	SK	Slovak Republic
CI	Côte d'Ivoire	KZ	Kazakhstan	SN	Senegal
CM	Cameroon	LI	Liechtenstein	SU	Soviet Union
CS	Czechoslovakia	LK	Sri Lanka	TD	Chad
CZ	Czech Republic	LU	Luxembourg	TG	Togo
DE	Germany	MC	Monaco	UA	Ukraine
DK	Denmark	MG	Madagascar	US	United States of America
ES	Spain	ML	Mali	VN	Viet Nam
FI	Finland	MN	Mongolia		

MUTANT GPIbα FRAGMENTS AND RECOMBINANT EXPRESSION THEREOF

Cross-Reference to Related Applications

This is a continuation-in-part of application Serial No. PCT/US91/00087, filed in the United States Receiving Office on January 4, 1991, which designates the Unites States and is a continuation-in-part of U.S. application Serial Nos. 07/613,083, filed November 14, 1990 and 07/460,674, filed January 4, 1990, said application Serial No. 07/613,083 being a continuation-in-part of said application Serial No. 07/460,674, which is itself a continuation-in-part of U.S. application Serial No. 07/121,454, filed November 17, 1987, and now abandoned.

Field of the Invention

This invention relates to polypeptides which are useful in the treatment of vascular disorders such as thrombosis. This invention further relates to the production by recombinant DNA-directed methods of pharmacologically useful quantities of the polypeptides of the present invention.

The term "hemostasis" refers to those processes which comprise the defense mechanisms of the body against loss of circulating blood caused by vascular injury. Processes which are normal as a physiologic response to vascular injury may lead in pathologic circumstances, such as in a patient afflicted with atherosclerotic vascular disease or chronic congestive heart failure, to the formation of undesired

thrombi (clots) with resultant vascular occlusion. Impairment of blood flow to organs under such circumstances may lead to severe pathologic states, including myocardial infarction, a leading cause of mortality in developed countries.

The restriction or termination of the flow of blood within the circulatory system in response to a wound or as a result of a vascular disease state involves a complex series of reactions which can be divided, for purposes of discussion, into two processes, primary and secondary hemostasis. Primary hemostasis refers to the process of platelet plug or soft clot formation. Platelets are non-nucleated discoid structures approximately 2-5 microns in diameter derived from megakaryocytic cells. Effective primary hemostasis is accomplished by platelet adhesion, the interaction of platelets with the surface of damaged vascular endothelium on which are exposed underlying collagen fibers and/or other adhesive macromolecules such as proteoglycans and glycosaminoglycans to which platelets bind.

Secondary hemostasis involves the reinforcement or crosslinking of the soft platelet clot. This secondary process is initiated by proteins circulating in the plasma (coagulation factors) which are activated during primary hemostasis, either in response to a wound or a vascular disease state. The activation of these factors results ultimately in the production of a polymeric matrix of the protein fibrinogen (then called "fibrin") which reinforces the soft clot.

Therapeutic drugs for controlling thrombosis have been classified according to the stage of hemostasis which is affected by the administration thereof. Such prior art compositions are typically classified as anticoagulants, thrombolytics and platelet inhibitors.

The anticoagulant therapeutics typically represent a class of drugs which intervene in secondary hemostasis. Anticoagulants typically have no direct effect on an established thrombus, nor do they reverse tissue damage. Associated with the use of existing anticoagulants is the hazard of hemorrhage, which may under some conditions be

greater than the clinical benefits otherwise provided by the use thereof. As a result, anticoagulant therapy must be closely monitored. Certain anticoagulants act by inhibiting the synthesis of vitamin K-dependent coagulation factors resulting in the sequential depression of, for example, factors II, VII, IX, and X. Representative anticoagulants which are used clinically include coumarin, dicoumarol, phenindione, and phenprocoumon.

Thrombolytics act by lysing thrombi after they have been formed. Thrombolytics such as streptokinase and urokinase have been indicated for the management of acute myocardial infarctions and have been used successfully to remove intravascular clots if administered soon after thrombosis occurs. However, the lysis effected thereby may be incomplete and nonspecific, i.e., useful plasma fibrinogen, in addition to fibrin polymers within clots, is affected. As a result, a common adverse reaction associated with the use of such therapeutics is hemorrhage.

A third classification, antiplatelet drugs, includes drugs which suppress primary hemostasis by altering platelets or altering or preventing their interaction with other circulatory system components. The present invention relates to antiplatelet drugs.

Reported Developments

Specific antiplatelet drugs operate by one of several mechanisms. A first example involves reducing the availability of ionized calcium within the platelet cytoplasm thereby impairing activation of the platelet and resultant platelet aggregation. Pharmaceuticals representative of this strategy include prostacyclin, and also Persatine® (dipyridamole) which may affect calcium concentrations by affecting the concentration of cyclic AMP. Numerous side effects related to the administration of these compounds have been reported. An additional class of antiplatelet drugs acts by inhibiting the synthesis of thromboxane A_2 within the platelet, reducing the platelet activation response. Non-steroidal anti-inflammatory agents, such as ibuprofen, phenolbutazone and napthroxane may produce a similar effect

by competitive inhibition of a particular cyclooxygenase enzyme, which catalyzes the synthesis of a precursor of thromboxane A_2. A similar therapeutic effect may be derived through the administration of aspirin which has been demonstrated to irreversably acetylate a cyclooxygenase enzyme necessary to generate thromboxane A_2. A third anti-platelet mechanism has involved the platelet membrane so as to interfere with surface receptor function. One such drug is dextran, a large branched polysaccharide, which is believed to impair the interaction of fibrinogen with platelet receptors that are exposed during aggregation. Dextran is contraindicated for patients with a history of renal problems or with cardiac impairment. The therapeutic ticlopidine is stated to inhibit platelet adhesion and aggregation by suppressing the binding of von Willebrand factor and/or fibrinogen to their respective receptors on the platelet surface. However, it has been found that ticlopidene possesses insufficient specificity to eliminate the necessity of administering large doses which, in turn, may be associated with clinical side effects.

The aforementioned pharmaceuticals are foreign to the body and may cause numerous adverse clinical side effects, there being no way to prevent such compounds from participating in other aspects of a patient's physiology or biochemistry, particularly if high doses are required. It would be desirable to provide for pharmaceuticals having such specificity for certain of the reactions of hemostasis, that they could be administered to patients at low doses, such doses being much less likely to produce adverse effects in patients.

An example of a pharmaceutical which is representative of a therapeutic that is derived from natural components of the hemostatic process is described in EPO Publication No. 317278. This publication discloses a method for inhibiting thrombosis in a patient by administering to the patient a therapeutic polypeptide comprised of the amino-terminal region of the α chain of platelet membrane glycoprotein Ib, or a subfragment thereof (hereinafter the α chain is referred to as glycoprotein Ibα or "GPIbα").

The present invention is directed to the provision of modified (including mutant) antithrombotic polypeptides patterned upon glycoprotein Ibα.

Summary of the Invention

In accordance with the present invention there is provided a polypeptide patterned on a fragment of wild type glycoprotein Ibα having a predetermined affinity for von Willebrand factor, said polypeptide having a modified sequence of amino acids relative to that of said fragment and an increased binding affinity, relative to said predetermined affinity, for von Willebrand factor ("vWF"). In comparison with the wild type form of fragments of GPIbα, the polypeptides of the invention exhibit a higher degree of therapeutic antithrombotic activity than the comparable wild type sequences.

The modified sequence of amino acids of a polypeptide of the invention, relative to that of GPIbα, may result from deletion of one or more amino acids (such as by mutation of an encoding DNA therefor), or from covalent labeling of GPIbα polypeptide or of a fragment thereof. A preferred modification results when, in comparison to a fragment of wild type GPIbα, one or more amino acid residues of said fragment are replaced with one or more amino acid residues found at the equivalent sequence positions of glycoprotein Ibα as isolated from one or more humans with platelet-type von Willebrand disease.

A preferred method for preparing polypeptides of the invention comprises the steps of:

(A) providing a DNA sequence encoding glycoprotein Ibα, or fragment thereof, in which one or more wild type codons thereof are replaced by codons specifying one or more amino acid mutations that confer upon the resultant expressed polypeptide enhanced binding affinity for von Willebrand factor relative to that of the comparable wild type sequence;

(B) inserting the DNA sequence so provided into a suitable plasmid or vector to create a construct comprising an expression plasmid or viral expression vector, said

construct being capable of directing the expression of said polypeptide in a cell;

(C) transforming a host cell with said construct; and

(D) culturing said transformed host cell under conditions that cause expression within the host cell of the resultant polypeptide.

The process may be practiced with, for example, DNA sequences that encode GPIbα, or a fragment thereof, in which one or more wild type codons thereof are replaced by codons specifying one or more amino acid mutations found in the glycoprotein Ibα DNA sequence of one or more patients having platelet-type von Willebrand disease.

Alternatively, an appropriate DNA sequence, encoding a GPIbα-derived polypeptide having increased binding affinity for vWF, may be provided by random mutagenesis of the wild type DNA sequence using a randomized population of oligonucleotides.

A further aspect of the invention provides for a method of treating or inhibiting thrombosis in a patient which comprises administering to such patient an effective amount of one or more therapeutic compositions of the invention, said compositions comprising a pharmaceutically acceptable carrier and one or more polypeptides of the invention.

Brief Description of the Drawings

Figure 1 is a graph showing the ability of mutant and wild type GPIbα fragments to bind to vWF in the presence of ristocetin.

Definitions

Unless indicated otherwise herein, the following terms have the indicated meanings.

Cloning Vehicle (Vector) - A plasmid, phage DNA or other DNA sequence which is able to replicate in a host cell, typically characterized by one or a small number of endonuclease recognition sites at which such DNA sequences may be cut in a determinable fashion for the insertion of heterologous DNA without attendant loss of an essential biological function of

the DNA, e.g., replication, production of coat proteins or loss of expression control regions such as promoters or binding sites, and which may contain a selectable gene marker suitable for use in the identification of host cells transformed therewith, e.g., tetracycline resistance or ampicillin resistance.

Plasmid - A nonchromosomal double-stranded DNA sequence comprising an intact "replicon" such that the plasmid is replicated in a host cell. When the plasmid is placed within a procaryotic or eucaryotic host cell, the characteristics of that cell may be changed (or transformed) as a result of the DNA of the plasmid. For example, a plasmid carrying the gene for tetracycline resistance (Tet^R) transforms a cell previously sensitive to tetracycline into one which is resistant to it. A cell transformed by a plasmid is called a "transformant."

Expression Plasmid - A plasmid into which has been inserted the DNA being cloned, such as the glycoprotein Ibα structural gene. The DNA sequence inserted therein may also contain sequences which control the translation of mRNA resultant therefrom, and contain restriction endonuclease sites which facilitated assembly of, and may facilitate further modification of, said expression plasmid. An expression plasmid is capable of directing, in a host cell, the expression therein of the encoded polypeptide and usually contains a transcription promoter upstream from the DNA sequence of the encoded structural gene. An expression plasmid may or may not become integrated into the host chromosomal DNA. For the purpose of this invention, an integrated plasmid is nonetheless referred to as an expression plasmid.

Viral Expression Vector - is similar to an expression plasmid except that the DNA may be packaged into a viral particle that can transfect cells through a natural biological process.

Table 1 shows the standard three letter designations for amino acids as used in the application.

TABLE I

Alanine	Ala
Cysteine	Cys
Aspartic Acid	Asp
Glutamic Acid	Glu
Phenylalanine	Phe
Glycine	Gly
Histidine	His
Isoleucine	Ile
Lysine	Lys
Leucine	Leu
Methionine	Met
Asparagine	Asn
Proline	Pro
Glutamine	Gln
Arginine	Arg
Serine	Ser
Threonine	Thr
Valine	Val
Tryptophan	Trp
Tyrosine	Tyr

Detailed Description of the Invention

As set forth above, the antithrombotic polypeptides of the present invention are based upon fragments of the natural occurring protein glycoprotein Ibα. For background purposes, there is set forth hereafter information concerning this protein and its role in hemostasis and thrombosis.

Description of the Role of GPIbα in Hemostasis and Thrombosis

The adhesion of platelets to damaged or diseased vessels occurs through mechanisms that involve specific platelet membrane receptors which interact with specialized adhesive molecules. One such platelet receptor is the glycoprotein Ib-IX complex which consists of a noncovalent association of two integral membrane proteins, glycoprotein Ib (GPIb) and glycoprotein IX (GPIX). GPIb, which is a two-chain molecule having an apparent molecular mass of approximately 160 kDa, is composed of a heavy (alpha, or "GPIbα") chain, having a molecular mass of approximately 145 kDa, linked by disulfide bonds to a light (beta, or GPIbβ) chain, having a molecular

mass of approximately 22 kDa. GPIb is an integral membrane protein and both the alpha- and beta- chains described above have transmembrane domains. The term "glycocalicin" refers to a soluble proteolytic fragment of the heavy (α) chain of GPIb that is generated by cleavage in a position close to the transmembrane domain of the molecule (Yamamoto, K. et al. Thromb. Res., 43, 41-55 (1986)). It is now clear that glycoalicin comprises most of the extracellular domain of the GPIbα from which it derives.

The adhesive ligand of the GPIb-IX complex is the protein von Willebrand factor ("vWF") which is found as a component of the subendothelial matrix, as a component of the α-granules secreted by activated platelets, and also as a circulating blood plasma protein. The actual binding site for vWF on the GPIb-IX receptor has been localized on the amino terminal region of the α chain of glycoprotein Ib, specifically, to within a fragment thereof having a molecular weight of 45,000 (45 kDa) and comprising approximately a His^{1}-Arg^{293} fragment of GPIbα. Binding sites exist also for vWF in the subendothelium of a damaged or diseased blood vessel, for example, on the collagen fibers, glycosaminoglycans or proteoglycans exposed therein, the vWF acting as a bridge between the damaged vessel and the now-adhering platelets.

GPIbα and vWF perform essential roles in normal hemostasis during vascular injury and are also of central importance in the pathogenesis of acute thrombotic occlusions in diseased blood vessels. Both of these roles involve the interaction of vWF with platelets (at GPIbα) which are induced to bind at the affected site, and are then crosslinked. It is believed that single platelets first adhere to a thrombogenic surface after which they become activated, a process involving major metabolic changes and significant morphological changes within the platelet. Activation is evidenced by the discharge of platelet storage granules containing adhesive substances such as additional quantities of von Willebrand factor, and the expression on the surface of the platelet of additional functional adhesive sites. Once activated, and as a part of normal hemostasis,

platelet "cells" become aggregated, a process which involves extensive crosslinking of the platelet cells with additional types of adhesive proteins.

It is believed that the interaction of multimeric vWF with glycoprotein Ib-IX complex (at GPIb(α)) results in platelet activation and facilitates the recruitment of additional platelets to a now growing thrombus. The rapidly accumulating platelets are also crosslinked (aggregated) by the binding of fibrinogen at platelet glycoprotein IIb-IIIa receptor sites, and possibly also by vWF at these sites, and/or at additional glycoprotein Ib-IX receptor sites. In addition, the glycoprotein IIb/IIIa receptor may also be involved in the formation of the initial monolayer of platelets. Of particular importance in this process is the multimeric and multivalent character of circulating vWF, which enables the macromolecule to effectively carry out its binding and bridging functions, for example, to GPIbα.

As stated above, these processes are normal as a physiologic response to vascular injury. However, they may lead in pathologic circumstances, such as in diseased vessels, to formation of undesired platelet thrombi with resultant vascular occlusion. Accordingly, inactivation or inhibition of the GPIbα receptors on the platelets of a patient would be of great medical importance for treating or inhibiting thrombosis. Accordingly, the present invention relates to the development of polypeptides which are effective in accomplishing the foregoing.

Other circumstances in which it is desirable to prevent deposition of platelets in blood vessels include the prevention and treatment of stroke, and to prevent occlusion of arterial grafts. Platelet thrombus formation during surgical procedures may also interfere with attempts to relieve preexisting vessel obstructions. The polypeptides of the invention are useful also in these circumstances.

The mechanisms responsible for triggering the binding of vWF to GPIbα <u>in vivo</u> have not yet been determined. Normally, GPIbα on platelets and vWF coexist in circulation, without any significant interaction occurring. Contact by vWF with exposed or damaged subendothelium triggers its binding

thereto and, possibly, vWF assumes an altered conformation (which is then capable of complex formation with GPIbα) when contacting a blood vessel wall. See Sakariassen et al., Nature, 279, 636-638 (1979); Stel et al., Blood, 65, 85-90 (1985); and Turitto et al., Blood, 65, 823-831 (1985). Conformational changes necessary for this binding may also be induced in GPIbα by contact of the platelet with other blood components or exposure of the platelet to high sheer stress in a damaged vessel. Moake et al., Blood, 71, 1366-1374 (1988).

The interaction between vWF and GPIbα can be demonstrated in vitro by several methods. Binding can be demonstrated in the presence of ristocetin, a glycopeptide antibiotic which may act by reducing excess negative charge density between the macromolecules. See Howard et al., Thromb. Diath. Haemorrh., 26, 362-369 (1971); and Coller et al., J. Clin. Invest., 60, 302-312 (1977). The interaction may also be triggered by the presence of the protein botrocetin, a component of certain snake venoms. Read et al., Proc. Nat'l. Acad. Sci. USA., 75, 4514-4518 (1978). The interaction between vWF and GPIb can also be enhanced by removing terminal (negatively charged) sialic acid carbohydrate residues from the vWF molecule. De Marco et al., J. Clin. Invest., 68, 321-328 (1981).

The amino acid sequence of the aforementioned amino terminal 45 kDa fragment of GPIbα (comprising residues His^{1}-Arg^{293} of the α polypeptide) has been reported by Titani, K. et al., Proc. Natl. Acad. Sci. USA, 84, 5610-5614 (1987). A complete cDNA encoding human GPIbα polypeptide has been determined by Lopez et al., Proc. Natl. Acad. Sci. USA, 84, 5615-5617 (1987). The gene for GPIbα has been cloned from a genomic cosmid library utilizing a partial cDNA clone as a probe, and its sequence, including introns, has been determined by Wenger, R.H. et al. Biochemical and Biophysical Research Communications, 156(1), 389-395 (1988). The GPIbα sequence predicted thereby consists of a 16 amino acid signal peptide, Met^{-16} through Pro^{-1}, followed by a 610 amino acid mature peptide or polypeptide region, His^{1} through Leu^{610}. The structure and properties of GPIbα are reviewed in Ruggeri,

Z.M., The Platelet Glycoprotein Ib-IX Complex, in Progress in Hemostasis and Thrombosis, vol. 10, p.3568, Coller, B.S. ed., W.B. Saunders Co., Philadelphia, 1991.

The Modified Polypeptides of the Present Invention

The terms "peptide" and "polypeptide" are used herein interchangeably.

This invention provides antithrombotic polypeptides patterned on fragments of wild type glycoprotein Ibα polypeptide. The polypeptides are effective in inhibiting activation and/or aggregation of platelets and adhesion of platelets to damaged or diseased vascular surfaces. The invention reflects the discovery that polypeptides which have a modified sequence of amino acids relative to the fragments of wild type GPIbα upon which they are patterned and which possess increased binding affinity for vWF, relative to that of said wild type GPIbα fragments, are effective to treat or inhibit thrombosis. Without being limited as to theory, it is believed that such modified polypeptides are effective because they inhibit binding of vWF to platelet membrane-bound GPIbα. Additionally, modified polypeptides of the invention (mutant polypeptides, as described below) can be expressed from recombinant bacterial or eucaryotic host cells.

A polypeptide of the present invention comprises an amino acid sequence which is patterned upon the amino acid sequence of wild type glycoprotein Ibα, but an amino acid sequence which is structurally different in one or more respects than the amino acid sequence of said wild type glycoprotein Ibα, said difference in structure being such that the polypeptide of the present invention has an increased binding affinity for vWF. According to the practice of the invention, a modified sequence of amino acids includes a difference in the sequence of amino acids of a fragment of glycoprotein Ibα polypeptide, compared to wild type sequence, without regard to how the difference is produced. Accordingly, examples of "modifications" with respect to one or more amino acid residues at one or more sequence positions in a polypeptide include deletions,

additions, substitutions of amino acids, and also the covalent labelling of amino acids present therein, or the addition of amino acids containing such labels, i.e., radicals or blocking groups which affect the properties of the amino acid residues so labelled.

In the practice of the invention, the term "wild type amino acid sequence" refers to the sequence of amino acids of a fragment of glycoprotein Ibα that is present in a large majority of humans. It is to be understood, however, that there are mutations in GPIbα, as isolated from certain persons, that do not affect significantly the interaction of GPIbα with vWF, for example, the Thr/Met^{145} dimorphism. Fragments of GPIbα containing such mutations are also to be considered wild type.

Additionally, it is understood that: (1) all references herein to glycoprotein Ibα fragments refer to GPIbα of humans, and that (2) GPIbα, or a fragment of GPIbα, as described herein, whether "wild type" or "modified", refers to a polypeptide that may or may not contain one or more additional functional groups added by a mechanism of post-translational modification in a cell, such as glycosyl or sulfate groups. Such groups may be added to polypeptide fragments of the invention by this mechanism or by an <u>in vitro</u> mechanism, including enzyme-catalyzed labelling or chemical labelling. It should be understood also that the invention encompasses biologically unimportant differences between the actual DNAs and polypeptides utilized in the practice of the invention and the structural sequences of amino acids or nucleotides thereof as reported herein.

As mentioned above, the present invention provides modified fragments of GPIbα by, for example, mutation or covalent labelling so that the resultant polypeptides bind to vWF with a greater affinity than the comparable fragments upon which they are patterned. Although GPIbα polypeptide fragments reflecting wild type GPIbα sequences can be used to inhibit binding of vWF to platelet-bound GPIbα in patients, it is desirable to identify polypeptides having increased affinity for vWF relative thereto which are therefore effective at lower doses. Provision of such polypeptides

having an increased binding affinity allows for more effective clinical treatments including the provision of an effective dose by a lower concentration of therapeutic, with the result that potential adverse clinical consequences such as possible immune response are minimized.

For the purpose of the invention, a binding affinity is considered increased if the modification increases the therapeutic utility of the polypeptide by either: (1) increasing the affinity of the polypeptide for vWF relative to that of the comparable wild type fragment of GPIbα upon which it is patterned by at least about 10%, as measured in a suitable in vitro or in vivo assay; or (2) achieving an equivalent amount of binding to vWF with about 10% less polypeptide; or (3) with respect to a particular amino acid sequence modification, achieving (1) or (2) above by use of the said modification in combination with one or more other "modifications" to the amino acid sequence. In preferred form, the antithrombotic therapeutics of the invention have an increased binding affinity of about 100% or more for vWF, and most preferably an increased binding affinity for vWF of about 5 fold or higher, compared to the wild type sequences of the GPIbα fragments upon which they are patterned.

Assays suitable for measuring "increased binding affinity" include (1) inhibition by GPIbα fragments of ristocetin-mediated binding of vWF to GPIbα on platelets; (2) inhibition by GPIbα fragments of botrocetin-mediated binding of vWF to GPIbα on platelets; (3) relative increase in ristocetin-mediated binding of vWF to immobilized GPIbα fragments (see Example 5 below); and (4) an increase in direct binding (without modulator) of vWF to immobilized GPIbα fragments following generally the procedure in Example 5 below. Trapani Lombardo, V. et al., J. Clin. Invest., 76, 1950-1958 (1985) provide procedures for immobilization of platelets useful in assays associated with techniques (1) and (2) above. The following is a detailed procedure whereby, for example, 96 well polystyrene microtiter plates can be coated with formalin-treated platelets, said treatment not interfering with GPIbα function on the platelets. Fifty μl of a solution containing poly-L-lysine (.01 mg/ml) in "PBS"

(phosphate buffered saline - about 1.55 gm sodium phosphate monobasic, 8.35 gm sodium phosphate dibasic, 61.41 gm NaCl, up to 7 liters, pH 7.2) is added to each well and incubated at room temperature for 30 minutes. The liquid content of the wells is removed. Fifty μl of platelet solution (approximately $5x10^7$/ml in PBS) is added to each well after which the plates are spun for 5 minutes at 2000 rpm (an IEC Co. model PR-6000 centrifuge) using very slow acceleration to avoid plate cracking. The plates are then washed with a solution of 2% (w/v) bovine serum albumin ("BSA") in PBS using 0.2 ml/well. The liquid contents of each well are then removed. Fifty μl of 2% formalin in PBS is then added to each well and incubated for 30 minutes at room temperature, after which the liquid is removed. To each well is next added 50 μl of 2% BSA in PBS. The wells are incubated in this solution for 1 hour at 37°C after which the liquid is removed. There follow 3 washes with PBS using 0.2 ml/well/wash. PBS is then added to the wells and the plates are used or stored at 4°C.

Additional terms used in connection with the description of modified polypeptides of the invention patterned on fragments of GPIbα are "equivalent sequence position" and "comparable wild type sequence", said terms being described aptly by way of examples. With respect to the His^1-Ala^{302} fragment of GPIbα containing a valine (mutant) residue at position 233, the "comparable wild type sequence" is the His^1-Ala^{302} fragment containing glycine (wild type residue) at position 233. "Equivalent sequence position" means that the substituted amino acid (for example, Val^{233}) occupies the same position in the new polypeptide (position 233) as in the amino acid sequence of GPIbα polypeptide from a patient having platelet-type von Willebrand disease from whom the mutation was identified, or that the substituted amino acid (for example, Val^{233}) occupies the same position in the new polypeptide that Gly^{233} occupied in a wild type polypeptide fragment. Thus, for example, the incorporation of a $Gly \rightarrow Val^{233}$ mutation into a modified polypeptide would not affect the positions of Lys^{231}, Gln^{232}, Val^{234} or Asp^{235}.

An important aspect of the invention is the recognition that certain amino acid substitutions, additions or deletions (compared to the wild type GPIbα sequence) can be made in fragments of GPIbα polypeptide to increase the binding affinity thereof for vWF, thereby making the resultant polypeptides more effective antithrombotics than the fragments of wild type GPIbα upon which they are patterned. Polypeptides of the invention including such substitutions, additions and deletions can be made by any method known in the art, including chemical synthesis of a polymer of amino acids or, for example, from expression of an encoding DNA. Increased binding affinity toward vWF can be similarly achieved by covalent labelling of certain wild type amino acid residues of GPIbα fragments.

The modified polypeptides of the invention are patterned on fragments of GPIbα that comprise all or part of the binding domain of GPIbα for vWF (the amino terminal residues 1-293 thereof), and may contain also additional GPIbα polypeptide sequence. Typically, the modified polypeptides of the invention represent fragments of GPIbα ranging in size from about 5 to about 500 amino acid residues. Generally, the polypeptides of the invention do not contain the transmembrane domain of GPIbα, said domain commencing at approximately residue 486 of GPIbα polypeptide.

~~There are~~ hereafter described representative types and species of the modified polypeptides of the invention. There are described also methods to ascertain the identity of additional of such types and species.

(A) Mutations Associated with Platelet-Type von Willebrand Disease

A principal discovery that is associated with the development of the invention is that certain amino acid substitutions, additions, or deletions effective in the practice of the invention are reflected in, or are suggested by, the amino acid sequences of GPIbα polypeptide as derived from human patients afflicted with platelet-type von Willebrand disease. Such amino acid sequence mutations are considered to be responsible for said affliction, which is

manifested by a prolonged bleeding time after injury or surgery. Other manifestations of the disease, reflective of the underlying defect, are selective loss from circulation of high molecular weight multimeric forms of vWF that are essential for hemostasis, and also mild thrombocytopenia.

As stated in Ruggeri, Z.M. and Zimmerman, T.S., Blood, 70(4), 895-904 (1987) at 896, "the term von Willebrand disease (vWD) defines a bleeding disorder that is heterogenous in its modalities of genetic transmission, clinical and laboratory manifestations, and underlying pathogenic mechanisms. Common to the different forms of the disease is that they all represent a genetic disorder, transmitted in an autosomal manner, which alters the structure, functions or concentration of vWF." Numerous separate types and subtypes of vWD have been determined based on phenotypic characteristics of the respective proteins.

One subtype of vWD is type IIB, identified, for example, by an amino acid mutation $Trp^{550} \rightarrow Cys^{550}$, located in the GPIbα binding domain of vWF. Ware, J. et al., Proc. Natl. Acad. Sci. USA, 88, 2946-2950 (1991). Another subtype of vWD, which is a relatively rare form of vWD, is termed either "pseudo" von Willebrand disease (Weiss, J. et al., N. Engl. J. Med., 306, 326-333 (1982)), or "platelet-type" von Willebrand disease (Miller, J.L. and Castella, A., Blood, 60, 790-794 (1982), Miller, J.L. et al., J. Clin. Invest., 72, 1532-1542 (1983)). The molecular basis for platelet-type vWD has been believed to be a structural change somewhere within the GPIb-IX complex - rather than in vWF as is typical of von Willebrand disease - that results in an increased affinity between platelets and circulating plasma vWF. See Miller, J.L. and Castella, A. (1982) and Weiss, J. et al. (1982). It has been reported that an amino acid substitution at residue 233 of glycoprotein Ibα polypeptide (glycine to valine) was linked to expression of platelet-type vWD in an afflicted family. Miller, J.L. et al., Circulation, 82(4), 2364 (1990) (Abstract); Miller, J.L. et al., Proc. Natl. Acad. Sci. USA, 88, 4761-4765 (1991). These reports are merely suggestive of the involvement of the gly→val mutation of GPIbα in the disease state since GPIbα exists, as previously discussed, in

a complex with GPIbβ and GPIX polypeptides. The reported genetic characterization, Miller, J.L. et al., did not analyze the amino acid sequences of GPIbβ or of GPIX in the afflicted family.

An important aspect of this invention is therefore the demonstration that platelet-type vWD is caused by modifications (mutations) of the amino acid sequence of GPIbα, such modifications leading to enhanced affinity for vWF. Using the residue 1-302 fragment of GPIbα as an example, it is demonstrated that fragments of GPIbα that include one or more such mutations have, relative to wild type GPIbα fragments, increased binding affinity for vWF, and hence, increased utility as antithrombotics.

Example 4 below provides an exemplary method whereby one or more mutations in GPIbα (such as Gly^{233}→Val), indicative of platelet-type vWD, can be incorporated into GPIbα fragments providing thereby polypeptides which have improved activity, relative to fragments comprising comparable wild type GPIbα sequence, as antithrombotics. Example 5 below demonstrates the improved antithrombotic utility (measured by increased affinity for vWF) of GPIbα fragment containing the Val^{233} mutation.

Example 2 below provides an exemplary method for producing a His^{1}-Ala^{302} fragment of GPIbα that contains the vWF binding domain thereof. Example 3 below demonstrates that this fragment, when expressed from recombinant mammalian cells (using pMW2 plasmid) possesses native tertiary conformation. Incorporation of the Val^{233} mutation into the residue 1-302 fragment produces a polypeptide fragment having properties (Example 5) characteristic of full-length GPIbα in individuals afflicted with platelet-type vWD, for example, increased affinity for vWF at low doses of ristocetin.

An additional mutation in glycoprotein Ibα that is associated with the platelet-type von Willebrand disease state is methionine to valine at GPIbα position 239. Russell, S.D. and Roth, G.J., Blood, 78(10) 281a (1991) Abstract. This invention includes within its scope antithrombotic polypeptides that comprise an amino acid sequence which corresponds to the amino acid sequence of

fragments of GPIbα that include all, or a part of, the binding domain of GPIbα for vWF (approximately residues 1-293 thereof), and which is modified in that the sequence includes one or more amino acids corresponding to those amino acid residues of GPIbα responsible, in affected individuals, for platelet-type von Willebrand disease. It is expected that polypeptide fragments incorporating more than one such modification will, in many cases, be more effective as antithrombotics than polypeptide fragments comtaining only one such substitution.

This invention provides therefore for polypeptide fragments of GPIbα which incorporate one or more mutations associated with platelet-type vWD phenotype and have therefore, relative to the wild type sequence, an increased binding affinity for vWF. Accordingly, the practice of the invention includes incorporation of Val^{239} into GPIbα fragments to produce antithrombotic polypeptides. By occupying the vWF receptor sites for GPIbα, the polypeptides demonstrate antithrombotic utility, that is, they prevent or inhibit platelets from participating in the processes which, under normal or pathological circumstances, lead to thrombus formation. Representative of processes involved in thrombus formation and which can be inhibited by the polypeptides of the invention are platelet adhesion, activation and aggregation.

It is important to note that, viewed from the perspective of designing new antithrombotic therapeutics, the principal characteristic of the polypeptides of the invention is the manifestation of increased binding affinity for vWF, irrespective of whether the amino acid substitution(s) responsible therefor is actually identified in one or more patients presenting platelet-type vWD. The methods described below are useful in the identification or creation of such other effective polypeptide sequences. In addition, GPIbα mutations associated with platelet-type vWD (or mutations having such similar effect) can be incorporated into fragments of GPIbα, thereby providing increased binding affinity for vWF relative to comparable wild type fragments on which they are patterned even though said amino acid

residue substitutions are not inserted into the polypeptide at the exact equivalent sequence positions that the residue mutations occupy in one or more GPIbα polypeptides from particular patients. It is understood that all such resulting polypeptides having similar therapeutic properties are within the scope of the invention.

In the preferred practice of the invention, the aforementioned antithrombotic polypeptides are made by a process of genetic engineering (recombinant DNA technology) which involves providing a DNA sequence that encodes a polypeptide containing one or more amino acid substitutions reflective of GPIbα as isolated from one or more patients with platelet-type vWD. The types of amino acid substitutions useful in the design of said polypeptides include also use of amino acid mutations from presently unidentified patients, and/or from presently unsequenced or unidentified mutations of patients so afflicted. Polypeptides of the invention may also be made by chemical synthesis (see, for example, Houghten, R.A. et al., Proc. Natl. Acad. Sci. USA, 82, 5131-5135 (1985), or by enzymatic digestion of GPIbα or of a fragment thereof.

(B) Mutation of the Residue 220-250 Subsequence of GPIbα Fragments to Substitute One or More Bulky Non-polar Amino Acids for One or More Semi-polar and/or Hydrogen Bonding Wild Type Amino Acid Residues Thereof

Gly^{233} and Met^{239} are semipolar amino acids, with methionine being capable, additionally, of hydrogen bond formation. Reference to the published amino acid sequence of GPIbα (Titani, K. et al., Proc. Natl. Acad. Sci. USA, 84, 5610-5614 (1987) shows that there are numerous other semipolar or hydrogen bonding-capable residues in the 220-250 subsequence of GPIbα located near residues 233 and 239, specifically Gln^{221}, Asn^{223}, Asn^{226}, Tyr^{228}, Trp^{230}, Gln^{232}, Thr^{240}, Ser^{241}, Asn^{242}, Ser^{245}, Gln^{247} and Cys^{248}. The present invention includes within its scope a polypeptide which contains within its sequence, in place of one or more of the aforementioned residues, one or more bulky non-polar amino acid such as, for example, valine, leucine, isoleucine, tryptophan, and

phenylalanine (capable of disrupting the functional roles of the aforementioned semipolar or hydrogen bonding-capable residues, thereby mimicking the likely effects of Val^{233} and Val^{239} in known platelet-type vWD cases). When incorporated into the polypeptides of the invention, such mutations define additional antithrombotic polypeptides.

With respect to replacement of Gly^{233}, it is very likely that all other amino acid residues, and in particular, charged or bulky residues would confer on the resultant modified polypeptide a higher affinity for vWF. The presence of glycine is known to disrupt α-helical regions within proteins. Replacement of glycine by other amino acid species is likely to substantially alter adjacent tertiary structure. See Chou, P.Y. and Fasman, G.D., Biochemistry, 13, 222-244 (1974). Based on the study of computer models of allowed energy conformations predicted for the residue 228-238 peptide region, Pincus, M.R. et al. have proposed a theory concerning the role of Gly^{233} in the structure and function of GPIbα. Biochemica et Biophysica Acta, 1097, 133-139 (1991). The authors suggest that Leu^{233}, Met^{233} and Phe^{233} would promote vWF-GPIbα interaction, although no peptide or recombinant polypeptides containing such mutations were made and then assayed to probe the nature of their interaction with GPIbα.

In connection with the aforementioned substitutions of bulky nonpolar residues for semi-polar residues and/or residues capable of forming hydrogen bonds, it is noted, for example, that the replacement amino acids need not be placed at the equivalent sequence positions that are targeted for replacement in order to be effective.

The following examples are representative of useful modifications of GPIbα polypeptide according to the practice of the invention:

(1) Replacement of either Ala^{238} or Thr^{240} with a bulky valine residue may prevent (by steric hindrance) Met^{239} from forming a hydrogen bond contact that could have been prevented by direct replacement with Val^{239}.

(2) Deletion of a target amino acid residue (such as Gly^{233} or Met^{239}) leading to polypeptides having, as respective examples, the following sequences

N-terminus . . . Gly^{232} . . . Val^{234} . . . C-terminus

N-terminus . . . Ala^{238} . . . Thr^{240} . . . C-terminus

(3) Addition of two or more additional amino acid residues, whether neutral or positively or negatively charged, that prevent (sterically) the formation of a hydrogen bond contact that would otherwise be made. For example, there may be created in the polypeptide sequence by site-directed mutagenesis, after wild type Gln^{232} and before Val^{234} the following sequence designed to disrupt the structural role of Gly^{233}: - $Val^{233'}$ - Gly^{233} - $Val^{233'}$.

Additionally, residues such as Met^{239} may be covalently labelled, such as by iodoacetamide to prevent the original side chain interactions (such as a hydrogen bond contact) of the wild type residue. Serine and threonine residues may be labelled similarly by acetylation with acetyl chloride.

(C) Random Mutagenesis to Produce Platelet-type von Willebrand Disease-Like Mutations

The technique of random mutagenesis, Hutchison, C.A. et al., Proc. Natl. Acad. Sci. USA, 83, 710-714 (1986) can be used to generate encoding DNA for GPIbα fragments with random codon changes. By sequentially focusing on consecutive series of 10 to 20 codons from which mutant series are generated, GPIbα-derived polypeptides (covering individually or in combination, the entire vWF binding domain) and containing one or more random mutations can be generated. Assay systems, suitable for screening large numbers of individual bacterial or eucaryotic clones to identify those expressing polypeptide fragments having increased binding affinity for vWF are described in Example 6.

(D) GPIbα Fragments Subject to Posttranslational Modification

The practice of the invention includes also GPIbα polypeptide fragments that are expressed from recombinant procaryotic (bacterial) and recombinant eucaryotic cells (such as mammalian cells). Typically, a major difference between fragments expressed from these two types of recombinant cells is the extent of posttranslational processing thereof. For example, polypeptides that are

normally sulfated and glycosylated by recombinant host mammalian cells (GPIbα as isolated from blood contains both of these modifications) undergo substantially less of such modification if expressed in bacterial cells. Accordingly, included as polypeptides of the invention are both glycosylated and nonglycosylated forms thereof, sulfated and non-sulfated forms thereof, and forms of GPIbα polypeptide fragment that were or were not subject to any other posttranslational modification characteristic of one or more types of host cell.

Glycosylation and sulfation may also be accomplished by enzymatic or chemical means in vitro.

It is also contemplated that any of the above strategies (A) to (D) can be used in any combination thereof so that, for example, one or more mutations from the GPIbα gene of one platelet-type vWD patient could be incorporated into a DNA encoding a therapeutic GPIbα fragment along with one or more mutations from the DNA of another such patient, or along with one or more mutations not derived from a patient, but otherwise predicted or determined to confer antithrombotic properties. Similarly, more than one such predicted or determined mutation can be combined in an encoding DNA for expression therefrom of an antithrombotic polypeptide.

Cloning of Mutant and Wild Type GPIbα Fragments

With regard to the cloning aspects of the invention, elements necessary for the practice of the invention are: (A) DNA sequences which encode a suitable fragment of GPIbα, such as the His^{1}-Ala^{302} encoding DNA sequence; (B) an expression plasmid or viral expression vector capable of directing in a cell the expression therein of the aforementioned fragment; and (C) a host bacterial or eucaryotic cell in which said expression may be effected.

The GPIbα polypeptide fragments so expressed are expected not to be secreted from host cells without attachment to the nascent GPIbα fragment of a signal peptide. Purification of proteins expressed therein and the extraction of pharmacologically useful quantities thereof is expected to

be more difficult than if the GPIbα fragment could be caused to be secreted into the culture medium of the host cells.

Accordingly, in the preferred practice of the invention there is provided a DNA sequence encoding a fragment of GPIbα for insertion into a suitable host eucaryotic cell in which there is inserted upstream from the encoding sequence thereof a DNA sequence encoding the GPIbα signal peptide. Signal peptides corresponding to other protein species may prove equally effective to cause the secretion of GPIbα. von Heijne, G., J. Mol. Biol., 184, 99-105 (1985). When attached to the amino terminal end of the GPIb(α) polypeptide-encoding sequence, the signal peptide causes the polypeptide to be recognized by cellular structures as a polypeptide of the kind to be processed for ultimate secretion from the eucaryotic cell, with concomitant cleavage of the signal polypeptide from the mature GPIbα polypeptide fragment. Fragments of GPIbα having increased binding affinity for vWF, and being less than about 50 amino acid residues in length, can be made more efficiently by chemical synthesis, than by cloning. Alternately, such fragments may be prepared to constitute, for example, one-fourth of a longer polypeptide expressed from a recombinant host cell, the longer polypeptide being next subject to proteolysis at a susceptible site to release the smaller polypeptide.

A wide variety of expression plasmids or viral expression vectors are suitable for the expression of the GPIbα polypeptide fragments. One factor of importance in the selection of an expression system is the provision of a high efficiency transcription promoter directly adjacent to the cloned GPIbα insert. Another factor of importance in the selection of an expression plasmid or viral expression vector is the provision of an antibiotic resistance gene marker therein so that continuous selection for stable transformant eucaryotic host cells can be applied.

Examples of suitable plasmids for use in the practice of the invention include pCDM8, pCDM8^{neo}, pcDNA1, pcDNA1^{neo}, pMAM^{neo} and Rc/CMV. Plasmids whose use in the practice of the invention is preferred include pCDM8^{neo}, pcDNA1^{neo}, pMAM^{neo} and Rc/CMV.

A DNA sequence encoding the GPIbα polypeptide, or a fragment thereof, may also be inserted into a plasmid or vector suitable for causing expression of the polypeptide in a bacterial system (see, for example, Cruz, M.A. et al., J. Biol. Chem., 267,1303-1309 (1992)).

There are several viral expression vector systems suitable for the practice of the invention including those based upon retroviruses and those based upon baculovirus Autographa californica nuclear polyhedrosis virus.

Representative host cells comprising permanent cell lines suitable for the practice of the invention include CHO-K1 Chinese hamster ovary cells, ATCC-CCL 61; COS-1 cells, SV-40 transformed African Green monkey kidney, ATCC-CRL 1650; ATT 20 murine pituitary cells; RIN-5F rat pancreatic β cells; cultured insect cells, Spodoptera frugiperda; or yeast (Sarcomyces), and also strains of E. coli.

Examples 1 and 2 below contain an explanation of preferred procedures used to express the GPIbα polypeptide or an amino terminal fragment thereof. Details of these procedures are found in Murata, M. et al., J. Biol. Chem., 266, 15474-15480 (1991), and published International Application No. PCT/US91/00087 (published on July 11, 1991 bearing International Publication No. WO91/09614).

Antibodies with Therapeutic Activity

Antibodies, and particularly conformation dependent antibodies, are powerful tools for analyzing the structure and function of macromolecules. By blocking macromolecular interactions, antibodies can also have important therapeutic utility.

Accordingly, this invention includes within its scope an antibody which is specific for the GPIbα polypeptide, or any fragment thereof, and which is made by a process which involves immunizing animals with a polypeptide that itself is patterned upon a fragment of GPIbα and contains one or more amino acid residues corresponding to platelet-type von Willebrand disease. This invention includes also within its scope an antibody made by immunizing with one or more other polypeptides of the invention.

Therapeutic compositions

One or more of the polypeptides of the present invention can be formulated into pharmaceutical preparations for therapeutic, diagnostic, or other uses. To prepare them for intravenous administration, the compositions are dissolved in water typically containing also one or more physiologically compatible substances such as sodium chloride. There results a solution having a pH, ionic strength, and osmotic potential compatible with therapeutic use (the range of potential solute concentrations being well known in the art, or readily determined), said water and physiologically compatible substances comprise a pharmaceutically acceptable carrier.

With respect to the therapeutic use of the polypeptides of the invention, the amount to administer for the prevention or inhibition of thrombosis will depend upon the affinity of the polypeptide for vWF in vivo, and/or for other macromolecules that participate in hemostasis and thrombosis in the body, on the lifetime of the polypeptide in the body, and on the severity with which the patient is subject to thrombosis. Said amount can be determined readily for any particular patient.

It is also within the practice of the invention to provide a therapeutic composition containing one or more of the polypeptides of the invention and also additional therapeutic substances. Such additional substances include heparin and other anticoagulants, aspirin or other antiplatelet drugs, or tissue plasminogen activator or other prefibrinolytic drugs.

The following Examples are representative of the practice of the invention.

Example 1

GPIbα (His^{1}-Leu^{610}) Expression
in Stable Mammalian Transformants

Production of the full-length mature GPIbα polypeptide (His^{1}-Leu^{610}) by expression from an encoding DNA therefor in host mammalian cells is described in published International Application No. PCT/US91/00087, (published on July 11, 1991,

bearing International Publication No. WO91/09614), and, in particular, Example 9 thereof. As described therein, an expression plasmid, pMW1, was created from which the His^{1}-Leu^{610} polypeptide can be expressed in CHO-K1 Chinese hamster ovary cells, or other mammalian cells. The GPIbα-encoding DNA sequence contains upstream from the residue 1-610 encoding sequence (or upstream from the residue 1-302 encoding sequence, see Example 2), a DNA sequence encoding the GPIbα signal peptide. When attached at the amino terminal end of the residue 1-610 polypeptide (or residue 1-302 fragment thereof, see Example 2), the signal peptide causes the polypeptide to be recognized by cellular structures as a polypeptide of the kind to be processed for ultimate secretion from the cell, with concomitant cleavage of the signal peptide from the mature GPIbα polypeptide.

Example 2

Expression of a His^{1}-Ala^{302} Fragment of GPIbα in Stable Mammalian Transformants

This example describes examplary conditions under which a DNA sequence encoding the fragment of mature GPIbα polypeptide having an amino terminus at His^{1} and a carboxy terminus at residue Ala^{302} thereof may be expressed, resulting in secretion from recombinant mammalian cells of the residue 1-302 GPIbα fragment. This fragment contains sufficient primary sequence information to be assembled in a host cell into a polypeptide possessing domains of tertiary structure present in native glycoprotein Ibα and possessing the biological activity thereof. (see Example 3 below and Murata, M. et al., J. Biol. Chem., 266, 15474-15480 (1991)). The fragment corresponds to the 45 kDa amino terminal fragment (His^{1}-Arg^{293}) of GPIbα that contains the vWF binding domain thereof, and which can be produced by tryptic digestion of GPIbα. See Vicente, V. et al., J. Biol. Chem., 263, 18473-18479 (1988), Vicente, V. et al., J. Biol. Chem., 265, 274-280 (1990).

Details of the procedure for expression of the fragment (see, generally, Murata, M. et al.) are found in published International Application No. PCT/US91/00087 and, in

particular, in Example 10 thereof. As described therein, an expression plasmid, pMW2, was created that is suitable for expression of the His^{1}-Ala^{302} polypeptide in mammalian cells.

It is expected that the expression of a polypeptide containing the amino acid sequence from approximately His^{1} to approximately Ala^{302}, and also additional GPIbα sequence on the carboxy terminal side of Ala^{302}, will also result in a polypeptide possessing the biological activity of the 45 kDa fragment.

Example 3

Demonstration of Native Tertiary Structure in the Polypeptide Produced by pMW1 and pMW2 Plasmids

The presence of GPIbα antigen in stable transformant cells (containing pMW1 or pMW2 plasmid) was demonstrated by applying cell lysates or culture medium from CHO-K1-containing dishes (both prepared as in Examples 9 and 10 of aforementioned International Application PCT/US91/00087) to nitrocellulose.

10 μℓ aliquots of lysate or culture medium were spotted onto nitrocellulose membranes (.45 micron pore size, Bio-Rad, Richmond, CA) and air dried. The membrane was then soaked with constant shaking for 2 hours at 22-25°C in "Blotto" (5 mg/mℓ fat-free dry milk, 0.25 mM phenylmethyl sulfonyl fluoride, 0.15 M NaCl in phosphate buffer pH 7.3), a protein blocking solution to inhibit nonspecific interaction.

The membrane was then incubated with native GPIbα conformation-requiring monoclonal antibody (5-10 μg/mℓ of antibody LJ-Ib1, see Vicente, V. et al., J. Biol. Chem., 263, 18473-18479 (1988) and J. Biol. Chem., 265, 274-280 (1990); Handa, M., J. Biol. Chem., 261, 12579-12585 (1986), or antibody LJ-P19, Murata, M. et al. (1991), for two hours at 22-25°C. After washing 3 times with Blotto, the membrane was transferred to a solution of ^{125}I-labelled rabbit anti-mouse IgG (0.08-0.16 mCi I^{125} per dot) and incubated for 2 hours at 22-25°C. The wash with Blotto was repeated 3 times prior to drying and making the autoradiograph (using Kodak AR film).

Cell extract and culture medium from untransformed CHO cells were used as controls. It was demonstrated (using LJ-Ib1 and LJ-P19 as primary antibody) that rIbα1 antigen and

rIbα2 antigen (produced by pMW1 and pMW2 transformants respectively) contain domains of tertiary conformation present in native GPIbα. Similar results were obtained using another conformation dependent anti-GPIbα monoclonal antibody, LJ-P3, Handa, M., J. Biol. Chem., 261, 12579-12585 (1986). See also Example 11 and Figure 4 of aforementioned International Application PCT/US91/00087.

Example 4

Expression of His^{1}-Ala^{302} GPIbα fragment, encoding valine at position 233 and/or at position 239 thereof in stable mammalian transformants

Site directed mutagenesis following the general procedure of Kunkel, T.A. et al., Methods Enzymol., 154, 367-383 (1987), and utilizing an appropriate oligonucleotide, was used to create a His^{1}-Ala^{302} polypeptide incorporating the Gly^{233}→Val mutation (the appropriate expression plasmid was designated pMW2/G233V and included a GGT→GTT mutation. See Murata, M. et al., J. Biol. Chem., 266, 15474-15480 (1991). A similar procedure can be used to incorporate separately or simultaneously the Met^{239}→Val mutation (using, for example, an ATG→GTG mutation).

Example 5

Ristocetin-Induced Binding of ^{125}I-vWF to Immobilized GPIb(α) Polypeptide Produced by pMW2 Plasmid (Gly^{233} versus Val^{233})

This example demonstrates that both the His^{1}-Ala^{302} polypeptide produced by CHO-K1 cells stably transformed with pMW2 plasmid or with pMW2/G233V plasmid (the Gly^{233} mutant) are functionally active. To perform the assays, a device used for the enzyme-linked immunofiltration technique (ELIFA) was adapted in combination with immobilization of recombinant pMW2 polypeptides. The 45 kDa GPIbα fragments (provided as respective culture media) were immobilized onto a nitrocellulose membrane (0.45 μ pore size) placed at the interface between a 96-well sample application plate and a vacuum chamber. Culture medium from nontransformed CHO cells was used as control. Commercially available filtration apparatus (Pierce Chemical Co., Rockford, IL) and pump materials (Miniplus 2, Gilson Co., Middleton, WI) were used.

See Murata, M. et al., J. Biol. Chem., 266, 15474-15480 (1991).

Immobilization of the 45 kDa fragments was accomplished by causing a 200 μl volume of culture medium (nonconcentrated) from the pMW2-transformed CHO cells to be vacuum-drawn through the nitrocellulose membrane over a 5 minute period. The protein binding capacity of the membrane was then saturated by passing through it three consecutive 200 μl aliquots of HEPES/BSA buffer, herein comprising 20 mM Hepes, pH 7.4, 150 mM NaCl, and 1% w/v bovine serum albumin (Calbiochem, La Jolla, CA).

After completion of the above procedure to minimize background caused by nonspecific interaction, a 50 μl volume of HEPES/BSA containing ^{125}I-vWF which had been preincubated therein with ristocetin (Sigma Chemical Co., St. Louis, MO) was vacuum drawn through the nitrocellulose membrane over a 5 minute period. Preincubation of the 50 μl aliquots was accomplished at room temperature for 30 minutes using various concentrations of ristocetin (0-2.0 mg/ml) and a specified amount of ^{125}I-vWF (2.0 μg/ml having a specific activity of about 10^9 cpm/mg).

The membrane was then allowed to dry and discs corresponding to the position of each application well were cut out and counted in a γ scintillation spectrometer (Crystal Multidetector RIA System, Packard Co., Burlingame, CA) to determine bound radioactivity. An autoradiograph of the membrane was also obtained before cutting out the discs in order to ascertain that there was no leakage of radioactivity from one well to another.

^{125}I-vWF radioactivity bound was determined as a function of the ristocetin concentration measured in the preincubation mixture (0-2.0 mg/ml) for control CHO cell extract, and for culture medium from pMW2 and pMW2/G233V transformed cells.

The increased affinity of the pMW2/G233V polypeptide compared to the pMW2 "wild type" polypeptide is demonstrated in Figure 1. Although binding to vWF at high (1.0-2.0 mg/ml) doses of ristocetin was essentially identical for pMW2 and pMW2/G233V polypeptides, at low doses of ristocetin (for example, 0.25 mg/ml) where binding by pMW2 polypeptide is

barely above the control value (about 10,000 cpm bound), approximately 25,000 cpm of ^{125}I-vWF are detected bound to immobilized pMW2/G233V polypeptide. An appropriate range of ristocetin concentrations may be determined for the testing of each modified polypeptide.

As a result of the above characterization of the functional properties of the His^{1}-Ala^{302} polypeptide, there is demonstrated the molecular basis of platelet-type von Willebrand disease.

Example 6

Mutagenesis of a fragment (residues 1-302) of GPIbα to create additional GPIbα-derived polypeptides having properties reflective of platelet-type von Willebrand disease

The following methods are representative of techniques which can be employed to (A) identify within the residue 1-302 sequence of GPIbα additional amino acids of the wild type sequence thereof involved in modulating binding of GPIbα to vWF; and/or (B) to create artificial GPIbα-derived polypeptide sequences with enhanced binding activity toward vWF.

Method 1 — Random mutagenesis of the residue 1-302 amino acid fragment of GPIbα to generate antithrombotic polypeptides

Using GPIbα DNA from plasmid pMW1 (Example 1) or from plasmid pMW2 (which encodes GPIbα amino acid sequence residues 1 to 302, Example 2) and random mutant oligonucleotides which will sequentially span the entire 302 residue amino acid sequence, novel variant DNA sequences can be constructed which encode variant GPIbα-derived polypeptides. Resultant potential therapeutic polypeptides can be screened for relative binding affinity (1) in direct binding assays for affinity for vWF, (if the mutation is particularly effective), or (2) in ristocetin-induced binding assays.

The success of random mutagenesis procedures is dependent upon the availability of binding assays which may be adapted to screen large numbers of mutant clones for expressed polypeptides having enhanced affinity for vWF. Procedures useful to screen extracts of large numbers of

bacterial clones are generally more efficient than similar procedures used to screen large numbers of recombinant mammalian clones. However, since "mammalian" polypeptides produced from recombinant bacterial host cells are often expressed as insoluble masses (inclusion bodies) it is necessary to provide procedures for the solubilization thereof. See, for example, Prior, C. et al., Biotechniques, 10, 66-73 (1992). Alternatively, it is desirable to express polypeptides that are soluble without such manipulations. Since formation of incorrect disulfide bonds (in or between individual polypeptides) is a difficulty associated with solubilizing proteins from inclusion bodies, it is preferred that random mutations be expressed within relatively short polypeptides having few cysteine residues, and therefore a reduced tendency for disulfide bonding. Alternatively, cysteine codons in the encoding DNA can be replaced by site-directed mutagenesis with inert codons such as those for serine or alanine.

Cruz, M. et al., J. Biol. Chem., 267(2), 1303-1309 (1992) disclose expression from bacterial cells and then solubilization (without denaturants) of the hydrophilic residue Gln^{221}-Leu^{318} fragment of GPIbα, said fragment containing only 2 cysteine residues. Since it may prove difficult to express the entire residue 1-302 polypeptide fragment from host bacterial cells without formation of incorrect disulfide bonds and concomitant inclusion body formation, the residue 221-318 subfragment thereof, or other similar-sized subfragment of the residue 1-302 fragment can be used to identify (screen) promising random mutations. For therapeutic use, such mutations may then be copied into a polypeptide comprising GPIbα amino acid sequence that is shorter or longer than the residue 221-318 subfragment, for example, the residue 1-302 fragment of GPIbα. The resultant polypeptide may, if necessary, be expressed from a recombinant mammalian host cell, Example 2, to provide native conformation. The appropriate size GPIbα DNA subsequence (for example, the encoding sequence for residues 221-318, Cruz, M. et al.) may be generated from pMW1 or pMW2 DNA using, for example, the polymerase chain reaction.

Random mutagenesis experiments can also be performed using GPIbα DNA constructs suitable for expression in mammalian cells such as those of Example 2, said expressed polypeptides appearing in stable soluble form in the culture medium.

Preparation of Oligonucleotides

Mutant oligonucleotides suitable for site directed mutagenesis protocols and spanning sequential 10 amino acid subdomains of the selected GPIbα sequence (for example, corresponding to amino acids 220 - 229, 230 - 239, 240 - 249 etc., within a residue 210-300 GPIbα fragment) can be generated using a procedure designed to yield a randomly mutagenized oligonucleotide population. Hutchison, C.A. et al., Proc. Natl. Acad. Sci., USA, 83, 710-714 (1986). The randomized GPIbα oligonucleotide is then hybridized, for example, to M13mp18 bacteriophage containing an appropriate GPIbα-derived insert (for loopout mutagenesis) to copy the mutation into a residue 210-300 encoding DNA sequence.

The method of Hutchison, C.A. et al. relies on automated synthesis of the oligonucleotide from the 3′ end. In the Hutchison procedure, a random oligonucleotide population suitable for causing permutation of the residues between, for example, positions 230 and 250 of the mature GPIbα polypeptide would be constructed as follows. The oligonucleotide corresponds to transcribed strand DNA. As the chain is then built stepwise by the nonenzymatic 3′→5′ addition of subsequent bases (comprising the part of the GPIbα sequence region to be surveyed), each of the four nucleoside phosphoramidite reservoirs (A,T,G,C) for oligonucleotide synthesis would be "doped" with a small amount of each of the other three bases. Incorporation of one of the "doping" nucleotides would result in a mutant oligonucleotide. The amount of doping can be adjusted to control results. The resultant randomized population of mutant oligonucleotides is then used in the standard site directed mutagenesis protocol (Example 4, see also Kunkel, T.A. et al., Methods Enzymol., 154, 367-383 (1987) to construct a pool of mutagenized GPIbα-fragment encoding DNA

sequences in M13mp18 corresponding to the GPIbα polypeptide residue 210-300 subfragment and suitable for subcloning into a bacterial expression system. This sequence may be used, in turn, in a mutagenesis procedure to generate a comparable mutant 1-302 fragment, or other GPIbα fragment, expressing the particular new random mutation.

It is possible to control the number of mutations per molecule by controlling the composition of the base mixtures. For example, it is possible to select for only single base pair substitutions or to select for molecules which have 2, 3, 4, or more mutations. The procedure developed by Hutchison, supra, typically employed solutions of each of the four bases in which approximately 1.5% impurity of each of the other three bases contaminates the original base solutions. Mutagenesis using this particular doped mixture resulted in roughly 41% of clones with no base substitutions, 40% with one, 15% with two, 3% with three and 0.7% with four (for target nucleotide sequences corresponding to 10 amino acids).

The resultant mutant M13mp18 populations are then subject to restriction, and the mutagenized DNA sequences are inserted into vectors or plasmids such as pET-3A (Rosenberg, A.H. et al., Gene, 56, 125-135 (1987)) for expression in host bacterial cells. Large scale screening of mammalian clones is generally more difficult than for bacterial clones. However, promising mutations identified in bacterial constructs may later be inserted into mammalian or other eucaryotic host cells for further testing or for commercial-scale polypeptide production.

The mutant clones can then be screened in GPIbα binding assays or in binding assays with GPIbα-specific monoclonal antibodies. Mutant clones having cell lysates which exhibit enhanced vWF binding or antibody response can be sequenced to determine the amino acid alteration(s) responsible for the mutant phenotype. In this way, a very systematic analysis of the residue 210-300 region of vWF subunit can be performed and mutations which enhance the binding of GPIbα to vWF can be identified. The process can be repeated, for example, for

the residue 1-100, and 100-200 subfragments of the residue 1-302 fragment of GPIbα.

Method 2 Random mutation of a targeted subdomain of the residue 1-302 sequence to develop therapeutic polypeptides

As previously mentioned, two residue positions within the residue 1-302 fragment of GPIbα polypeptide had been suspected of providing a molecular basis for platelet-type von Willebrand disease. These sites are at amino acid positions 233 (Gly→Val) and 239 (Met→Val).

Since the known mutations indicate a primary sequence subfragment wherein platelet-type von Willebrand disease properties can be successfully generated, and are also proximal to each other, random mutagenesis of the DNA corresponding to the short peptide sequence directly adjacent to these particular residues (i.e., residues 220-250) would be emphasized.

Method 3 Mutagenesis of specific target amino acids within the residue 220-250 subfragment of GPIbα to develop additional therapeutic polypeptides

The two known mutations which correlate with platelet-type von Willebrand disease result in replacement of a wild type codon, encoding a semipolar amino acid, with a codon corresponding to the nonpolar and bulky residue valine. Representative further target amino acid residues, substitution for which is predicted to yield mutant polypeptides having platelet-type vWD properties and resultant antithrombotic therapeutic utility include Gln^{221}, Asn^{223}, Asn^{226}, Tyr^{228}, Trp^{230}, Gln^{232}, Thr^{240}, Ser^{241}, Asn^{242}, Ser^{245}, Gln^{247} and Cys^{248}. Suitable replacements for the above amino acid residues include, as examples, isoleucine, valine, leucine, tryptophan and phenylalanine.

Cloned GPIbα polypeptide constructs reflecting known platelet-type vWD mutations may also be subject to the above mentioned random mutagenesis procedures and then screened for restoration of normal binding function such as, for example, having a normal response in a modulator-induced vWF binding assay. For example, it may be demonstrated that a particular

"reversion" mutation proximal to residue 233 would compensate for, and nullify the effect of the original $Gly^{233} \rightarrow Val^{233}$ mutation. The associated DNA sequence can then be determined to identify the relevant counteracting amino acid. Such procedures can be used to give important further evidence as to which other residue positions in the wild type amino acid sequence are important modulators of GPIbα binding.

Screening of mutant GPIbα-derived polypeptides for enhanced vWF binding activity

There is hereafter presented an effective method to screen randomly mutagenized residue subfragments of GPIbα polypeptide for enhanced vWF binding activity, and resultant enhanced therapeutic utility.

To perform the assays, a device used for the enzyme-linked immunofiltration assay technique (ELIFA), Pierce Chemical Co., Rockford, IL, can be adapted in combination with immobilization of the mutant GPIbα-derived polypeptides to be tested. It is considered most efficient to initially test the effect of mutant codons on GPIbα polypeptides expressed from bacterial constructs and to then copy potentially useful mutations (using, for example, mutagenesis in M13mp18 vehicle) into a mammalian expression construct. High levels of mutant GPIbα polypeptides corresponding to mutant DNA sequences can be expressed from, for example, pET-3A type bacterial expression plasmids. Mutant polypeptides are expected to constitute a major portion of host E.coli cell lysates and can be readily screened for vWF affinity.

Accordingly, site directed mutagenesis can be performed following the general procedure of Example 4 using as template in M13mp18 the GPIbα-encoding sequence for the residue 210-300 subfragment of GPIbα. For the oligonucleotide pool, oligonucleotides each having, for example, randomly mutagenized residue 230 to 240 sequences are used.

The mutagenized population of M13mp18 constructs can be cloned into pET-3A plasmids after which the expression plasmids can be transformed into host E.coli cells, for example, ampicillin sensitive strain BL21(DE3), Novagen Co., Madison, WI. Strain BL21(DE3) contains a gene for T7 RNA

polymerase for high efficiency transcription. Preparation of mutant polypeptide extracts from E.coli BL21(DE3) for screening follows the procedure of Prior, C. et al., if inclusion bodies develop. Otherwise, a procedure such as that of Cruz, M. et al. would be appropriate, or other procedures adaptable from the known art. Polypeptides corresponding to residues 1-100, 101-200, or 150-250 can be expressed similarly, and then tested as follows.

Resultant extracts of expressed mutant GPIbα polypeptide subfragments are immobilized following the manufacturer's instructions onto a nitrocellulose membrane (0.45μ pore size) using 96-well sample application plates (Easy-Titer® ELIFA System, Pierce Co., Rockford, IL) and a vacuum chamber. Commercially available pump materials can be used (see Example 5). The apparatus is suitable for screening large series of clone lysates in an ELIFA or dot blot system and allows also quantitative transfer of sample fluids to underlying microtiter wells without cross contamination.

Immobilization of the GPIbα polypeptides is accomplished by causing a suitable volume of GPIbα fragment solution, such as 200 μl, (including also 8 M urea if resuspended inclusion body pellet material is used) to be vacuum-drawn through the individual wells to the nitrocellulose membrane over a 5 minute period. Several 200 μl volumes of Hepes-buffered saline are then drawn through the membrane to remove urea.

The protein binding capacity of the membrane is then saturated by passing through it three consecutive 200 μl aliquots of HEPES/BSA buffer herein comprising 20 mM Hepes, pH 7.4, 150 mM NaCl, and 1% w/v bovine serum albumin (Calbiochem, La Jolla, CA).

After completion of the above procedure to minimize background caused by nonspecific interaction, a 50 μl volume of HEPES/BSA containing ^{125}I-vWF (following the procedure of Example 5) which had been preincubated therein with ristocetin (at approximately 0-0.5 mg/ml) can be vacuum drawn through the nitrocellulose membrane again over a 5 minute period. See Example 5 above, for suitable preincubation conditions. Multiple plates may be used to screen at different ristocetin concentrations.

The membrane is then allowed to dry and discs corresponding to the position of each application well are cut out and counted in a γ scintillation spectrometer to determine bound radioactivity. The counting process may be facilitated by scanning the developed autoradiogram in a densitometer to digitize the intensity of developed spots. As long as the autoradiogram is not excessively overdeveloped, beyond the linear region of response, useful qualitative results are obtained.

An alternate procedure to derive from individual host E.coli BL21 (DE3) clones an impure extract which can be screened in immunoblot or dotblot procedures is as follows. A large set of individual E.coli colonies carrying separate randomly mutagenized GPIbα residue 210-300 inserts is picked and grown overnight as separate cultures. The cultures are then diluted 1:100 and grown to an OD_{600} of 1.0. GPIbα fragment synthesis is induced by adding isopropyl-β-d-thiogalactopyranoside (IPTG), U.S. Biochemicals, Cleveland, OH, to 5 mM and continuing growth for approximately 2.5 hours. The cells are harvested by centrifugation for 1 minute at 10,000 g and then washed and repelleted (at 10,000 g) 3 times with phosphate buffered saline (0.14 M NaCl, 0.1 M Na_2HPO_4 pH 7.0). The bacterial pellet is then solubilized by boiling for 10 minutes in a buffer comprising 0.01 M NaH_2PO_4, 10 mM Na_2EDTA, 1% (w/v) sodium dodecylsulfate, pH 7.0. The incubation is continued for 2 hours at 60°C in the presence also of 10 mM dithiothreitol (DTT). Suitable volumes (such as 200 μl) of such extracts can be used directly in ELIFA apparatus or dot immunoblot analyses. Prior to adding ^{125}I-vWF to the plate several rinses of Hepes-buffered saline are washed through the wells. A concentration of ristocetin should be selected (such as 0.-0.5 mg/ml) that is sufficiently low that the ristocetin-mediated difference is discernable. For example, in Figure 1 (see also Example 5), it is necessary to go well below 1 mg/ml of ristocetin to resolve the enhancing activity of the 233 mutation.

GPIbα-derived polypeptides from colonies representing the most intense response are selected for confirmation

(rescreening) of enhanced binding using methods such as those described above.

Clones which confer enhanced positive responses in these systems are then subjected to standard DNA sequencing procedures to identify the GPIbα gene mutations responsible for the mutant properties. The appropriate mutations may be copied into a GPIbα DNA sequence within a plasmid suitable for expression in CHO-K1 cells, followed by further characterization therefrom.

We claim:

1. A polypeptide patterned on a fragment of wild type glycoprotein Ibα having a predetermined affinity for von Willebrand factor, said polypeptide having a modified sequence of amino acids relative to that of said fragment and an increased binding affinity, relative to said predetermined affinity, for von Willebrand factor.

2. A polypeptide according to Claim 1 wherein said modified sequence comprises one or more amino acid residues of said fragment replaced with one or more amino acid residues found at the equivalent sequence positions of glycoprotein Ibα, as isolated from one or more humans with platelet-type von Willebrand disease.

3. A polypeptide according to Claim 1 patterned on a fragment of glycoprotein Ibα beginning approximately at residue 1 (histidine) thereof and ending at approximately residue 302 (alanine) thereof.

4. A polypeptide according to Claim 1 wherein said modified sequence comprises valine at position 233 and/or valine at position 239.

5. A polypeptide according to Claim 1 wherein said modified sequence comprises at least one fewer amino acid residue in said fragment.

6. A polypeptide according to Claim 1 wherein said modified sequence comprises covalent labelling of one or more amino acid residues of said fragment.

7. A polypeptide according to Claim 1 prepared by mutagenesis of a DNA sequence.

8. A polypeptide according to Claim 1 that is subject to posttranslational modification.

9. A glycosylated and/or sulfated polypeptide according to Claim 1.

10. A process for producing from DNA encoding glycoprotein Ibα, or a fragment thereof, a biologically active polypeptide which process comprises the steps of:
(A) providing a DNA sequence encoding glycoprotein Ibα, or fragment thereof, in which one or more wild type codons thereof are replaced by codons specifying one or more amino acid mutations that confer upon the resultant expressed polypeptide increased binding affinity for von Willebrand factor relative to that of the comparable wild type sequence;
(B) inserting the DNA sequence so provided into a suitable plasmid or vector to create a construct comprising an expression plasmid or viral expression vector, said construct being capable of directing the expression of said polypeptide in a cell;
(C) transforming a host cell with said construct; and
(D) culturing said transformed host cell under conditions that cause expression within the host cell of the resultant polypeptide.

11. A process according to Claim 10 in which step (A) thereof comprises:
(A) providing a DNA sequence encoding glycoprotein Ibα, or a fragment thereof, in which one or more wild type codons thereof are replaced by codons specifying one or more amino acid mutations found in the glycoprotein Ibα DNA sequence of one or more patients having platelet-type von Willebrand disease.

12. A process according to Claim 10 for generating a biologically active mutant polypeptide patterned upon wild type glycoprotein Ibα, or a fragment thereof, said polypeptide having relative to wild type glycoprotein Ibα, or said fragment thereof, an increased binding

affinity for von Willebrand factor, said process comprising the steps of:

(A) providing a population of oligonucleotides corresponding to one or more glycoprotein Ibα-encoding DNA subsequences, and containing therein random mutations within one or more of the codons within said subsequences;

(B) using the resultant population of mutant oligonucleotides in a mutagenesis procedure with a glycoprotein Ibα or glycoprotein Ibα fragment-encoding DNA sequence as template, thereby creating a random population of mutagenized sequences;

(C) inserting the resultant mixture of mutagenized glycoprotein Ibα or glycoprotein Ibα fragment-encoding DNA sequences into plasmids or vectors thereby creating a population of expression plasmids or viral expression vectors;

(D) inserting the resultant population of expression plasmids or viral expression vectors into suitable host cells;

(E) screening individual colonies or cultures of resultant host cells for expression of glycoprotein Ibα-derived polypeptides having properties reflective of platelet-type von Willebrand disease;

(F) having determined the DNA sequence of the glycoprotein Ibα insert in a colony or culture of a host cell expressing glycoprotein Ibα-derived polypeptide having said reflective properties;

(G) expressing the determined DNA sequence, or a DNA sequence which is constructed to reflect the changes identified in the determined sequence, in a host cell;

(H) isolating the mutant glycoprotein Ibα-derived polypeptide produced thereby.

13. A DNA sequence encoding a polypeptide according to Claim 1.

14. A DNA sequence according to Claim 13 encoding the fragment of glycoprotein Ibα polypeptide having an amino terminus at approximately residue 1 (histidine) and a carboxy terminus at approximately residue 302 (alanine), or a subfragment thereof, in which one or more codons thereof are replaced by mutant codons corresponding at equivalent sequence positions to codons as isolated from the DNA of one or more humans with platelet-type von Willebrand disease.

15. An expression plasmid or viral expression vector containing a DNA sequence according to Claim 13.

16. An expression plasmid or viral expression vector according to Claim 15 containing a DNA sequence encoding a fragment of mutant glycoprotein Ibα polypeptide, said encoding DNA containing one or more codons specifying one or more amino acid sequence mutations found in glycoprotein Ibα polypeptide as determined from one or more patients having platelet-type von Willebrand disease, said plasmid or vector being suitable for replication in a host cell and directing expression therein of said fragment.

17. A recombinant eucaryotic or procaryotic host cell transformed with an expression plasmid or viral expression vector according to Claim 15.

18. An antibody which is specific for glycoprotein Ibα polypeptide, or a polypeptide comprising a fragment thereof, said antibody being made by a process of immunizing animals with a polypeptide according to Claim 1 and then isolating the antibodies generated thereby.

19. A therapeutic composition which is effective in treating or inhibiting thrombosis which comprises
(A) a pharmaceutically acceptable carrier; and
(B) a polypeptide according to Claim 1.

20. A method of treating or inhibiting thrombosis in a patient which comprises administering to such patient an effective amount of one or more therapeutic compositions according to Claim 19.

21. A method of inhibiting adhesion, activation or aggregation of platelets which comprises adding to a solution containing platelets an effective amount of one or more therapeutic compositions according to Claim 19.

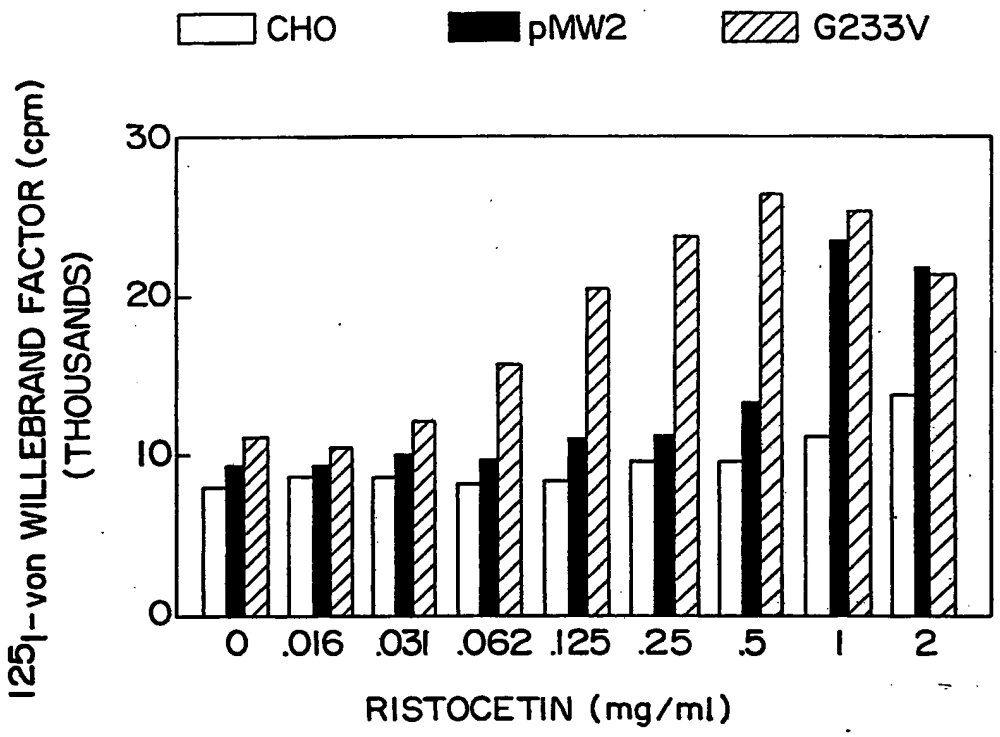
CHO
pMW2
G233V
125I-von WILLEBRAND FACTOR (cpm)
(THOUSANDS)
30
20
10
0
0
.016
.031
.062
.125
.25
.5
1
2
RISTOCETIN (mg/ml)

FIG. 1

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US93/01734

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :Please See Extra Sheet.
US CL :530/350, 388.25, 389.3; 514/12; 424/85.8; 536/23.1
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 530/350, 388.25, 389.3; 514/12; 424/85.8; 536/23.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS
search terms: GPIB or Glycoprotein-Ib and antibody

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	J. Biological Chemistry, Vol. 261, No. 27, issued 25 September 1986, Handa et al., "The von Willebrand Factor-binding Domain of Platelet Membrane Glycoprotein Ib", pages 12579-12585, see entire document.	1-21
Y	Science, Vol. 252, issued 21 June 1991, Waldmann, "Monoclonal Antibodies in Diagnosis and Therapy", pages 1657-1661, see entire document.	1-21
Y, L	TibTech, Vol. 11, issued February 1993, Harris et al., "Therapeutic antibodies - the coming of age", pages 42-44, see entire document.	1-21

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be part of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 28 April 1993	Date of mailing of the international search report 13 ... 1993
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. NOT APPLICABLE	Authorized officer DONALD E. ADAMS, PH.D. Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US93/01734

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Proc. Natl. Acad. Sci. USA, Vol. 84, issued August 1987, Titani, et al., "Amino acid sequence of the von Willebrand factor-binding domain of platelet membrane glycoprotein Ib", pages 5610-5614, see entire document.	1-21
Y	Methods in Enzymology, Vol. 154, issued 1987, Kunkel et al., "Rapid and Efficient Site-Specific Mutagenesis without Phenotypic Selection", pages 367-383, see entire document.	1-21
Y	EP, A, 0 317 278 (Zimmerman et al.) 24 May 1989, see entire document.	1-21
Y	Proc. Natl. Acad. Sci. USA, Vol 83, issued February 1986, Hutchison et al., "A complete library of point substitution mutations in the glucocorticoid response element of mouse mammary tumor virus", pages 710-714, see entire document.	1-21
Y	J. Biological Chemistry, Vol 265, No. 1, issued 05 January 1990, Vicente et al., "Identification of a site in the alpha chain of platelet glycoprotein Ib that participates in von Willebrand Factor binding", pages 274-280, see entire document.	1-21
Y	Proc. Natl. Acad. Sci. USA, Vol. 82, issued 15 August 1985, Houghten, "General method for the rapid solid-phase synthesis of large numbers of peptides: Specificity of antigen-antibody interaction at the level of individual amino acids", pages 5131-5135, see entire document.	1-21
Y	J. Biological Chemistry, Volume 266, No. 25, issued August 1991, Murata et al. "Site-directed Mutagenesis of a soluble Recombinant fragment of Platelet Glycoprotein Ib-alpha Demonstrating Negatively Charged Residues Involved in von Willebrand Factor Binding", pages 15474-15480, see entire document.	1-21
Y	J. Biological Chemistry, Volume 267, No. 2, issued 15 January 1992, Cruz et al., "Functional Analysis of a Recombinant Glycoprotein Ib-alpha Polypeptide Which Inhibits von Willebrand Factor binding to the Platelet glycoprotein Ib-IX complex and to collagen", pages 1303-1309, see entire document.	1-21

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US93/01734

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Biochema et Biophysica Acta, Vol. 1097, issued 1991, Pincus et al., "Conformational energy analysis of the substitution of Val for Gly 233 in a functional region of platelet GPIb-alpha in platelet-type von Willebrand disease", pages 133-139, see entire document.	1-21
Y	Proc. Natl. Acad. Sci. USA, Vol. 88, issued June 1991, Miller et al., "Mutation in the gene encoding the alpha chain of platelet glycoprotein Ib in platelet-type von Willebrand disease", pages 4761-4765, see entire document.	1-21
Y	J. Clin. Invest., Vol. 72, issued November 1983, Miller et al., "von Willebrand Factor Binds to Platelets and Induces Aggregation in Platelet-type but Not Type IIb von Willebrand disease", pages 1532-1542, see entire document.	1-21
Y	Blood, Vol. 60, No. 3, issued September 1982, Miller et al., "Platelet-Type von Willebrand's Disease: Characterization of a New Bleeding Disorder", pages 790-794, see entire document.	1-21
Y	Proc. Natl. Acad. Sci. USA, Vol. 88, issued April 1991, Ware et al., "Identification of a point mutation in type IIb von Willebrand disease illustrating the regulation of von Willebrand factor affinity for the platelet membrane glycoprotein Ib-IX receptor", pages 2946-2950, see entire document.	1-21
Y	Blood, Vol. 70, No. 4, issued October 1987, Ruggeri et al., "von Willebrand factor and von Willebrand Disease", pages 895-904, see entire document.	1-21
Y	J. Clin. Invest., Vol. 76, issued November 1985, Trapani et al., "Independent modulation of von Willebrand Factor and Fibrinogen binding to the Platelet Membrane Glycoprotein IIb/IIIa Complex as Demonstrated by Monoclonal Antibody", pages 1950-1958, see entire document.	1-21
Y	Biochemical and Biophysical Research Communications, Vol. 156, No. 1, issued 14 October 1988, Wenger et al., "Structure of the human blood platelet membrane glycoprotein Ib-alpha gene", pages 389-395, see entire document.	1-21

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US93/01734

A. CLASSIFICATION OF SUBJECT MATTER:
IPC (5):A61K 37/00, 39/00; C07H 15/12, 17/00; C07K 3/00, 13/00, 15/00, 17/00